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VARIATIONS IN THE COMPLEMENT CONTENT OF SERUM AND PLASMA.*†

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That doubt as to the presence or absence of complement in the circulating plasma should still exist after several years of observation and repeated experiment, seems, at first sight, somewhat extraordinary. That there is a difference of opinion on the subject is evident from the diverse statements which, in turn, are based on the observation of experiment, published by different authors. Gengou,¹ over 10 years ago, satisfied at least himself that plasma does not contain a complementary body capable of reactivating specific sera *in vitro*. Since this time a moderate number of contributions have appeared relative to the subject; a relatively small proportion of these authors substantiating Gengou's observations, the majority stating their belief in the erroneousness of his conclusions.

The most recent article upon this subject is that by Addis,² who, basing his opinions on the results of his experiments upon cock's plasma and serum, states unqualifiedly that complement is present in substantially equal quantities in both plasma and fresh serum. He refers briefly to the work of Hermann, Domery, Sweet, Hewlett, Lourt, Schwartz, and others, all of whom, with the exception of Hermann, have been unable to corroborate Gengou's findings.

The reasons for such diverse opinions are due to the fact that the methods adopted for the estimation of complement quantitatively and qualitatively are liable to afford possibilities of error which are of the greatest importance. The simplest and most

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¹ *Ann. de l'Inst. Pasteur*, 1901, 15, p. 232.

² *Jour. Infect. Dis.*, 1912, 12, p. 208.

satisfactory means of estimating the complement content of serum and the one most commonly employed in routine experiment, as in the Bordet-Gengou reaction, is that which measures the activating property of a serum on amboceptor, especially as this is evidenced in the production of hemolysis of sensitized red blood cells. For general purposes such a method of determining the quantity of complementary body leaves little to be desired. If, however, we attempt to employ this method of measuring the complement contained in the fluid portion of uncoagulated blood, i.e., plasma, as has customarily been done, we find that a serious error may be encountered. This is due to the fact that it is probably impossible so to wash the erythrocytes used as the indicator that no other substance present in the blood from which they are obtained is present in the suspension.

It is quite possible, by means of the employment of paraffined tubes, albolene- and petrolatum-protected syringes and needles, and rapid chilling and centrifugalization, to procure a plasmatic fluid even from guinea-pigs' blood which is free from thrombo-kinase, or at least to limit its production to such minute proportions that fibrin formation will not occur even though the fluid be placed in contact with unprotected glass surfaces. This fact is well recognized by those experimenting on the cultivation of tissues *in vitro*. It is further noted by these observers that it is not necessary that leukocytes be added to the plasma in order that clotting may follow; simply the introduction of a small number of connective tissue cells into such a fluid is almost instantly followed by the deposition of fibrin in radiating lines from the point of application. Similarly we find that the addition of washed erythrocytes to fluid plasma is rapidly followed by the formation of a coagulum.

It appears, therefore, that sufficient thrombo-kinase to induce fibrin formation is present, closely associated with the cells, in erythrocyte suspensions such as are employed in complement-determining hemolytic reactions. If this be true, it appears possible that some body bearing a relationship to complement similar to that which thrombo-kinase bears to fibrin may likewise be present in such suspensions. I am of opinion that complement as it is

generally known and recognized in fresh serum does not exist as a staple constituent of normal plasma and I have attempted to prove this to be the case by means of the experiments described in this paper. I believe, furthermore, that the diverse opinions expressed by various investigators are explained chiefly at least by the experimental error just mentioned.

It will be noted that the evidence produced in the following pages, and on which this view is based, is both direct and indirect in character. Certain experiments appear to prove directly that complement is not present in plasma, while those which demonstrate the variations in the complement content in one and the same serum specimen if the latter be subjected to different conditions, especially in so far as this refers to temperature and time, should be looked upon as being almost equally suggestive.

In this paper I will confine myself to a study of the factors determining the development of complement, reserving the important question of the cause of the natural deterioration of serum in this respect for a later contribution.

TECHNIC.

The experiments upon which this paper is based were all performed with full-grown guinea-pigs, weighing from 450 to 550 gms. In procuring material the animals were anesthetized (with ether) and the blood withdrawn directly from the heart. Following removal, the blood was kept for varying lengths of time at different temperatures such as are mentioned in the text and tables. Rapid chilling, in those experiments demanding such treatment, was accomplished by means of exposure to a mixture of ice and common salt for several minutes, but not long enough to freeze the blood. Standard-sized centrifuge tubes were employed in all instances for receiving blood, quantities varying from 2.5 to 5.0 c.c. of blood being placed in each. This fact is noted, since the volume of blood exposed to variations in temperature is of importance inasmuch as smaller quantities are more rapidly affected by the temperature of the surrounding medium, water, air, etc. As a general rule from 3 to 5 c.c. were removed from each pig examined.

Immediately before use, all specimens were placed in the centrifuge and subjected to a speed of from 5,000 to 7,000 revolutions for from one to three minutes depending on the difficulty with which the serum was obtained clear.

In procuring plasma, paraffined centrifuge tubes placed in a freezing mixture were employed to receive the blood, which was obtained in a 5 c.c. all-glass syringe, using a large bore needle, the whole being heavily coated with vaseline.

The hemolytic series employed throughout has been the "sheep-rabbit" system, the amboceptor having in all cases an extremely high titer. Unless otherwise stated the total quantity of fluid employed in each experiment was 1.0 to 1.25 c.c., being made up with 0.85 per cent sodium chlorid solution. The suspension of sheep's erythrocytes employed was approximately 0.15 per cent, the mixtures being prepared in bulk so that the possible error which might arise from an unequal distribution of cells might be excluded.

INTERPRETATION OF TABLES.

The accompanying tables have been prepared in a more or less uniform manner; the horizontal line of figures stands for the corresponding numbers of drops of serum, which unless otherwise stated, have been diluted one to two, each drop having a value of approximately $1/40$ th c.c. The relative degree of hemolysis obtained is described by means of the employment of numerals instead of the customary collection of plus and minus signs. Complete hemolysis occurring during the time limit set for the experiment has been signified by the numeral 6; absence of hemolysis is shown by zero; while intervening stages of lysis have been designated by means of the intervening numerals.

EXPERIMENTS.

Tables 1, 2, and 3 give the results of observations on the available complement content of serum when the blood from which it is obtained is exposed to different degrees of temperature. It will be noted that the maximum amount of demonstrable complementary body is obtained in blood kept at body temperature, the minimum amount being found in blood at 0° C.

These experiments, in addition to drawing attention to the fact just mentioned, show that the amount of complement available for hemolytic purposes is somewhat greater even when the blood is kept under most favorable circumstances at the end of one hour than at the end of 15 minutes. It is worthy of note also that the amount of complement developed in the serum of different animals

TABLE 1.
ACTIVATING POWER OF GUINEA-PIGS' SERUM UNDER DIFFERENT CONDITIONS AS
REGARDS TEMPERATURE.

		3*	6	7	8	10
Guinea-pig 1.....	37° 1 hr., 5° 23 hrs....	3	4	4	5	6
Guinea-pig 2.....	37° 1 hr., 5° 23 hrs....	3	(Broken)	4	6	6
Guinea-pig 1.....	0° 1 hr., 5° 23 hrs....	1.5	1.5	2	4	4.5
Guinea-pig 2.....	20° 1 hr., 5° 23 hrs....	1.5	1.5	2	4.5	6

*Serum diluted 1 to 2

TABLE 2.
ACTIVATING POWER OF GUINEA-PIGS' SERUM PRESERVED AT DIFFERENT TEMPERATURES.

		2	4	5	7	9
Guinea-pig 3.....	1 hr 0°.....	0	2	3	4.5	6
Guinea-pig 3.....	1 hr. 20°.....	0	4.5	5	6	6
Guinea-pig 3.....	1 hr. 37°.....	6	6	6	6	6
Guinea-pig 4.....	1 hr. 0°.....	0	2	3	4.5	6
Guinea-pig 4.....	1 hr. 20°.....	0	4.5	5	6	6
Guinea-pig 4.....	1 hr. 37°.....	5	6	6	6	6
Guinea-pig 5.....	1 hr. 0°.....	0	3	4	6	6
Guinea-pig 5.....	1 hr. 37°.....	5	6	6	6	6

TABLE 3.
ACTIVATING POWER OF GUINEA-PIGS' SERUM PRESERVED AT DIFFERENT TEMPERATURES.

		2	3	4	5	7
Guinea-pig 6.....	37° 1 hr., 5° 18 hrs....	4.5	6	6	6	6
Guinea-pig 6.....	37° 15 min., 5° 18½ hrs..	3	4.5	5	6	6
Guinea-pig 7.....	37° 1 hr., 5° 18 hrs....	4	6	6	6	6
Guinea-pig 7.....	37° 15 min., 5° 18½ hrs..	2	4	5	6	6
Guinea-pig 6.....	5° 19 hrs.....	1.5	3	4	5	6
Guinea-pig 7.....	5° 19 hrs.....	2	3	4	5	6

is very nearly absolutely constant in amount. This fact I have sought to emphasize in a previous publication.¹ As might well be expected, it is found that although the development of complement proceeds very slowly at lower temperatures, the longer sera are stored in the ice-chest the more nearly does the complement content of different specimens tend to become equalized.

¹ *Jour. Infect. Dis.*, 1911, 8, p. 427.

The same feature of complement variation depending upon temperature is demonstrated by an experiment in which equal quantities of sera (four drops of a one-in-three dilution) from the same animal, but exposed to different temperatures, were tested with varying quantities of hemolytic body and a constant volume of erythrocytic suspension. By this time it was shown that serum preservation at a temperature of 37° for one hour rendered three drops of an amboceptor dilution sufficiently active, whereas serum procured from blood kept at 20° required the addition of eight drops of the same dilution of amboceptor containing serum to induce complete hemolysis.

While it has not been possible for the writer to prove the absolute absence of complement in plasmatic fluid from the guinea-pig, the following experiments, similar examples of which have been frequently repeated, were carried out, which prove that the amount developing can be markedly limited. It must be stated, however, that the various technical steps must be very carefully carried out. Otherwise, even the relative absence of complement, as evidenced by the accompanying experiments, cannot be demonstrated.

TABLE 4.
PROTOCOL SHOWING RELATIVE ACTIVATING POWER OF GUINEA-PIG SERUM AND PLASMA.

	2	4	5	6
Serum 1	3	5	6	6
Serum 2	3	6	6	6
Serum 3	3	5	6	6
Plasma 1	0	0	4	5
Plasma 2	0	0	4	5
Plasma 3	0	1	4	5

Serum prepared as follows: Clotted at 38° 15 minutes; chilled; centrifugalized.

Plasma procured as follows: Paraffin method, chilled, centrifugalized.

Total time of experiment, 20 minutes.

Serum and plasma used undiluted.

If the quantity of serum barely sufficient to bring about complete hemolysis under the conditions of the experiment, namely 1 c.c., be accepted as a unit, it will be noted that a similar quantity of plasma contains an amount of complement so small that no hemolysis whatever occurs.

The following series of experiments also demonstrate the relative absence of complement in blood preserved at a temperature below that of the body.

TABLE 5.
COMPARATIVE ACTIVATING POWER OF SERUM FROM THE SAME ANIMAL SUBJECTED TO
DIFFERENT TEMPERATURES.

		3	5	6
Guinea-pig 15.....	Clotted 37° 1 hr.	6	6	6
Guinea-pig 15.....	Whipped 20° 35 min.	3	6	6
Guinea-pig 15.....	Whipped, chilled, 2 hours.....	2.5	4.5	6
Guinea-pig 15.....	Diluted with saline, 1 to 8, 0° 30 min.	1	2	0
Guinea-pig 15.....	Clotted 45° 1 hr.	6	6	6

Experiments were also employed in which coagulation was prevented by means of sodium citrate. Results seemed to show that, if anything, the development of the complementary body was hastened by this method, this result being due, it is assumed, to the action of the citrate upon the white blood cells.

In view of the circumstantial evidence in favor of the development of complement in the form in which it is usually considered, subsequent to the removal of the blood from the body, and in view of the difficulty experienced in proving by the direct method the absence of complement in plasma as obtained by ordinary methods, I have employed what must be termed the indirect method of demonstrating the presence of complement. I have already mentioned my reasons for believing that this method ought to be productive of more positive results. The experiments to be enumerated seem to justify this contention.

If the presence of complement in plasmatic mixtures, as demonstrated by myself and numerous other observers, be due, as I believe, to the interaction of some substance present in the erythrocyte mixture and a complementogenic body in the blood fluid, it appeared to me that it should be possible to maintain this complementogenic substance intact if the blood fluid were exposed to the action of a specific antigen and in the absence of hemocellular products. For this purpose, guinea-pig plasma was mixed with an excess of *B. typhosus* antigen and antityphoid amboceptor derived from an immune rabbit. Following incubation for one

hour there was added to the mixture a quantity of "anti-sheep-rabbit" serum and washed sheep's erythrocytes, with results as detailed in Table 6.

TABLE 6.
BORDET-GENGOU REACTIONS EMPLOYING GUINEA-PIG SERUM AND PLASMA.

CONTROL SERIES					
Antigen	Specific Amboceptor	Guinea-Pig Serum*	Hemolysin	Cells	Result
3 drops	2	3	2 units (4C)	25 per cent	o
3 drops	3	4	2 "	25 " "	o
3 drops	4	5	2 "	25 " "	o
4 drops	5	6	2 "	25 " "	o
TEST SERIES					
Antigen	Specific Amboceptor	Plasma†	Hemolysin	Cells	Result
4 drops	4	1	2 units	25 per cent	6‡
4 drops	4	2	2 "	25 " "	6
4 drops	4	3	2 "	25 " "	4
4 drops	4	4	2 "	25 " "	6
4 drops	4	5	2 "	25 " "	6
4 drops	4	6	2 "	25 " "	6

* Diluted 1 to 2.

† Undiluted.

‡ Complete hemolysis at the end of 15 minutes.

This experiment and other similar ones appear to be capable of interpretation in but one way, namely, that complement in a form available for binding in the presence of antigen and specific amboceptor does not exist in plasma as procured from guinea-pigs' blood; it appears furthermore, that by the addition of a suspension of washed erythrocytes, some body is added which renders the plasma active at least in so far as its hemolytic power is concerned. It is but just to note that similar results are not obtained in all instances; I believe, however, that, in dealing with a material which is as difficult to procure as guinea-pig plasma, a few positive results are of more importance than a number of negative findings.

In all my experiments in which plasma has been added to a mixture of normal salt solution and either red blood cells or bacterial antigen, fibrin formation has consistently occurred. This fact is mentioned in view of the apparent importance attached to this phenomena by Addis.¹

¹ *Jour. Infect. Dis.*, 1912, 10, p. 208.

DISCUSSION.

It is my belief that complement as we are accustomed to consider it, in ordinary *in vitro* experiments, does not exist as such in the circulating plasma, but that a body which may be termed complementogen is present in practically constant amounts. This complementogen is rendered active only as a result of the liberation of some substance, probably similar to thrombo-kinase, which is produced chiefly by white blood cells.

These views are based on the facts observed and recorded above; namely, that the amount of complement demonstrable in serum varies with the length of time and the temperature at which the blood is kept, thus suggesting that it is a development following removal from the body; the more so since a temperature of 37.2° , or slightly higher, is more uniformly followed by a rapid production of the body than if a lower temperature be employed. There is no reason for believing, according to this hypothesis, that complement may not be produced as occasion may arise within the body, owing to the interaction *in vivo* of the two substances just mentioned. In order to harmonize Addis' results in experimentation on cock's serum I suggest the possibility that inasmuch as fowl's blood submits to relatively greater insult without coagulum formation, the complementogenic substance is acted upon with free complement formation during the time consumed in preparing the fluid. In other words, it may be that the complement-inducing substance is liberated in a shorter period of time than that necessary for the formation of a sufficient quantity of thrombo-kinase to bring about clotting.

SUMMARY AND CONCLUSIONS.

The maximum complement content of a given specimen of serum depends upon the temperature at which the blood is kept following removal from the body. The more nearly this temperature approaches that of the body the greater is the amount of complement developed and the more rapidly does it appear.

The maximum complement content of healthy animals of the same species is remarkably constant in amount.

Following the maximum development of the complementary

body there occurs some change, the nature of which has not been discussed in this paper, resulting in the reduction of the amount of complement available for hemolytic experiments. This depreciation in activating power of the serum occurs more rapidly at higher than at lower temperatures, being practically absent at temperatures of 0° C.

In the ordinary hemolytic experiments employed in the estimation of complement content of plasma, a relatively small amount of complement is demonstrated. The author has not, however, been able to prove the absence of this body in plasma by the employment of this method.

By means of the Bordet-Gengou complement binding reaction it is possible to prove that no complement susceptible to fixation in the presence of an excess of specific amboceptor and bacterial antigen is present in plasma.

From a consideration of the above facts the author concludes that complement is not a normal constituent of animal plasma but that there is present a pre-complement or "complementogen" which, when acted on by or in combination with some substance produced under stimulation of the blood, by certain body cells including the leukocytes, forms the finished complementary product.

From a practical point of view these experiments demonstrate that the serum of guinea-pigs, unless they be the subjects of acute infections, may be depended on to contain a constant amount of complementary body available for serum reactions, granted that the blood from which it is procured is preserved in a definitely standardized manner especially in so far as this refers to temperature and time. Conversely, blood kept in haphazard manner cannot be depended upon for employment in complement-binding reactions.

I have found that if, on removal from the body, the blood be placed at 37° C. for a period of from one-half to one hour, and subsequently placed in the ice-chest at a temperature of about 2° C. it may be employed with but little likelihood of experimental error arising therefrom during a period of from 24 to 48 hours.